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Introduction

Prostate cancer is the most common non-skin cancer among men in industrialized countries (1). The causes of prostate cancer, however, remain largely unknown, with age, race, and family history being the only established risk factors (2). The prostate gland has historically been considered the prototype of an androgen-dependent organ. However, there is evidence that estrogens may induce mitosis of both normal and malignant prostatic epithelial cells in many species, including humans (3,4).

In humans, 16α-hydroxyestrone (16α-OHE1) and estriol are biologically significant estrogens, and their activity can contribute to the overall expression of estrogenic action. Hydroxylation of estrone at the 16α-position, one of the two major and mutually exclusive biotransformation pathways of the estrogens, leads to estrogen metabolites with estrogenic activity (5-7) and genotoxic characteristics (8). The other main pathway for estrogen metabolism is via hydroxylation at the C-2 position, producing 2-hydroxyestrone (2-OHE1), a derivative that has virtually no estrogenic activity (6, 7, 9,10). In addition to the estrogenic effects of the 16α-hydroxylated metabolites, in *in-vivo* models, 16α-hydroxylation of estrone was associated with increased spontaneous incidence of tumors (11).

There is evidence that estrogen metabolism is related to breast cancer risk. Most case-control studies examining these metabolites have shown higher levels of estrone 16α-hydroxylation in breast cancer cases than in healthy controls, particularly for postmenopausal women (12-15). Similarly, two

prospective studies investigating the role of estrogen metabolism as predictor of breast cancer found that study participants with elevated 2-OHE1 /16α-OHE1 ratio (highest tertile) had a 40% reduction in breast cancer risk compared with those in the lowest tertile (16, 17).

In the present case-control study we examined the association between prostate cancer and estrogen metabolism. Specifically, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16α-hydroxylation would be associated with a decrease in prostate cancer risk.

Material and Methods

Study Subjects. We conducted a case-control study of incident, primary, histologically confirmed prostate cancer cases in Erie and Niagara counties, NY, USA (the PROMEN study). All participants provided informed consent; the Human Subjects Review Board of the University at Buffalo, School of Medicine and Biomedical Science and each of the participating hospitals approved procedures for protection of human subjects in the study. Prostate cancer patients were men between 45 and 85 years of age. Each prostate cancer case was enrolled in the study and urine was collected soon after diagnosis and before starting any cancer treatment. In addition, because the major focus of the study was the relation of estrogen metabolism to prostate cancer risk, patients on hormonal treatment (current or in the six months prior the diagnosis), or with known metabolic diseases affecting the endocrine profile (i.e., hypogonadism, hyperadrenalism) were excluded. Those affected with chronic or acute liver

diseases were also excluded because of their potential influence on estrogen metabolism. Patients with a previous history of cancer (except of non-melanoma skin cancer) were excluded as well. In addition, because we used driver's license records to identify controls aged 35-65, cases in that age range were also required to have a driver's license. To exclude latent prostate carcinomas that cannot be distinguished from those that would not progress to clinical disease (real latent carcinoma) and those detected in a very early phase of their progression, the present study included only patients with clinically apparent disease (stage B and greater by the staging system proposed by Catalona -18). To standardize the stage of the disease across the hospitals, a screening form developed in the context of the PROMEN study was completed by a trained nurse case-finder using the hospital pathology records. The forms were then reviewed, together with the hospital records, by Dr. Muti, the principal investigator of the study.

Every year, out of a total number of 690 incident prostate cancer cases detected in Erie and Niagara Counties, 450 were identified by the five major hospitals in Buffalo (New York State Cancer Registry). Urologists from two of those major hospitals agreed to fully collaborate with the Promen study and almost all prostate cancer cases were recruited at the Department of Veterans Affairs Medical Center of Western New York Health Care System (VAMC) and the Kaleida Health System (Buffalo General Hospital).

In the course of the study period, from December 1998 to April 2001, 504 prostate cancer cases were identified. Of these 504, 163 met eligibility criteria,

and were approved by the urologists and invited to join the PROMEN study. Of these 163, 50 refused to participate. Thus, among the eligible participants, 70% (113/163) of the subjects participated in the study. Seventeen prostate cancer cases did not provide morning spot of urine thus the present analysis is conducted on 96 subjects.

All prostate cancer cases were adenocarcinomas; 84% showed clinical or imaging evidence of the disease, with the tumor confined within the prostate gland (Stage B). Sixteen per cent of patients had tumors that extended through the gland invading the capsule (Stage C), among those, two patients had distant metastasis.

Control subjects were matched on place of residence (first four digits of the zip code). This matching criterion was introduced to reduce, at least in part, potential systematic differences between prostate cancer cases and controls subjects related to social and life-style factors. Those controls between 35-65 years of age were selected from a list of individuals holding a New York State driver's license and residing in Erie and Niagara Counties. Those aged 65 and over were selected from the rolls of the Health Care Finance Administration. Eligibility criteria for control subjects were the same as for cases. We excluded men on hormonal treatment (current or in the six months prior to the contact), or affected with metabolic or endocrine diseases. Participants with a previous history of cancer (except of non-melanoma skin cancer) were excluded as well. Because there is high prevalence of latent prostate carcinoma in men over age 50 (19, 20), we measured Prostate-Specific Antigen (PSA) in all blood samples

obtained from controls. Controls with a PSA value higher than 4ng/ml were excluded from the control group according to the criterion proposed by the American Cancer Society Prostate Cancer Detection Project (21) until the completion of diagnostic procedures to determine their true case-control status. We identified 8 prostate cancer cases as a result of the PSA determinations in control subjects.

During the study period, 1,373 potential controls were contacted. One hundred and seventy nine of these potential candidates were deceased and 115 were too ill to participate, 293 were not eligible, and we were not able to contact 273 individuals (wrong address, and wrong telephone number). Three hundred and seventeen of the remaining 513 subjects (60%) were enrolled and interviewed. Thirteen men did not provide morning spot of urine, thus the present analysis includes 304 participants.

Hormonal Determinations. For standardization purposes, morning spot urine was collected between 7:00 A.M. and 9:00 A.M. from all participants. The time at specimen collection was recorded. Samples were kept in -80°C freezers until biochemical determinations.

Stored urinary samples from prostate cancer cases and related controls were handled identically and randomly located in the laboratory runs. All laboratory personnel were blinded with regard to case-control status.

Analyses of 2-OHE1 and 16α-OHE1 were performed using a competitive solid-phase enzyme immunoassay (IMMUNA CARE Corporation, Bethlehem, PA). The urinary estrogen metabolites are found mostly as glucuronide

conjugates and require the removal of the sugar moiety before recognition by the monoclonal antibodies. A mixture of β-glucuronidase and arylsulphatase (glusulase from *H. Pomatia*, Sigma Chemical Co., St. Louis, MO) was used for this purpose. The enzyme digest was then neutralized. Assay incubation time was 3 hours at room temperature. The assay was read kinetically using a Molecular Devices Thermomax plate reader (Molecular Devices, Sunnyvale, CA) and the data were analyzed using SoftMax EIA Application software (Molecular Devices). Both assays have been shown to allow 100% recovery of metabolites with serial dilution and "spiking" of exogenous estrogens into urine samples. The EIA kits have previously been evaluated for validity and reproducibility and the values for each metabolite were compared with values obtained by gas chromatography-mass spectrometry (22-24). As a measure of reproducibility, two laboratory control samples and one sample from the manufacturer were included in all assays; and their values had to fall within two standard deviations from the mean of a continuous Levy-Jennings control plot. In addition, for 10% of the samples duplicates were included twice not identified to the laboratory performing the assay. All samples, standards and controls were assayed in triplicate. Samples that were not within 10% of each other were reassayed. Any sample that was too concentrated or diluted was reassayed at half concentration or 2-4 diluition, respectively. Intra -assay coefficients of variation for 2-OHE1 and 16α-OHE1 were 3.6% and 3.8%, respectively. Interassay coefficients of variation were 5.9% and 10.2%, respectively.

Statistical Analysis

2-OHE1 and 16α-OHE1 urinary levels were standardized by the total urinary creatinine. We used unconditional logistic regression to obtain the odds ratios of prostate cancer in relation to estrogen metabolites and their ratio. The independent variables of interest were 2-OHE1, 16α-OHE1 by tertiles of urine concentration and the 2-OHE1/16α-OHE1 ratio. We based the cutoff points for each tertile on the distribution of the estrogen metabolites in controls. We identified age, weight, waist-to-hip ratio, race and smoking, as potential covariates according to their potential biologic relevance and logistic regression was used to control for these covariates. In the initial regression model, we examined all variables. Age, weight, waist-to-hip ratio, race and smoking did not substantially modify the results. None of the potential covariates was a confounder of the association between prostate cancer and estrogen metabolites and their ratio. Nevertheless, we included them in further analysis to provide fully adjusted estimates for comparison with those reported in the published literature, in particular with the previous studies on hormones and prostate cancer risk.

We report here the odds ratio and its 95% confidence interval of prostate cancer risk per one unit change in the transformed value. Tests of significance for the continuous variables in the logistic regression models were also used to examine linear trend.

Results

Characteristics of the study population are reported in Table 1. Prostate cancer cases were more likely than control subjects to be slightly younger and heavier and to have higher waist-to-hip ratio and lower education. In a descriptive analysis on control subjects (Table 2) urinary levels of estrogen metabolites did not significantly differ by age strata, by racial groups or waist-to-hip ratio. Lighter subjects had significantly higher levels of 2-OHE1 than heavier men. Current and former smokers had higher concentrations of the estrogen metabolites, with the highest level of 16α-OHE1 in current smokers.

In Table 3, we show data on prostate cancer risk in relation to tertiles of estrogen metabolites concentrations, their ratio, and their 95% confidence intervals. There was a 20% odds reduction in the highest tertile of the 2-OHE1, however the confidence interval included unity. Conversely, there was increased risk for the highest tertile of 16α-OHE1 in both the crude and the adjusted point estimates. The odds ratio for the continuous variable was 3.98 (95% CI: 1.01-16.01) for each unit change in the logarithmically transformed value (p for trend = 0.05).

2-OHE1/16α-OHE1 ratio was associated with a reduction in odds ratios for prostate cancer across tertiles. Odds ratios for the second and third tertiles were 0.87 (95% CI: 0.49-1.54), and 0.60 (95% CI: 0.33-1.11), respectively, with a test for trend of p=0.05.

Conclusions

This study appears to indicate that the estrogen metabolism pathway favoring 2-hydroxylation over 16α-hydroxylation is associated with a reduced risk of prostate cancer risk. To our knowledge this is the first study investigating the effects of estrogen metabolism on prostate cancer risk and the first observation supporting the potential protective role of estrogen metabolites at low biological activity in prostate cancer development.

Epidemiological studies on the effects of estrogens in relation to prostate cancer risk, in particular serum estrone and estradiol have provided conflicting results (25-27). The inconsistency of results may be related to differences in control selection and/or in specimen collection (i.e., control of the sources of hormone variability such as circadian rhythm). It may also be that the relevant measure is not the serum level of estrone and estradiol but rather the estrogen metabolites that we measured in urine.

Serum concentrations of unconjugated estriol and 16α-OHE1 are low relative to estrone and estradiol (28-29) but their biological impact may be significant because of their lack of affinity for the sex hormone binding globulin (30). In addition, Fishman and colleagues (31, 32) have noted that 16α-OHE1 can uniquely bind to amino groups on the estrogen receptor, histones, and DNA. First, it is bound as reversible Schiff base then it followed by spontaneous rearrangement to yield a product in which the steroid is covalently linked resulting in a persistent estrogenic responses until the receptor is degraded. In circumstances of comparable hormone secretion, therefore, estradiol metabolism

shifted towards production of 16α-OHE1 could produce a hyperestrogenic milieu, while a predominance of 2-hydroxylation could produce hypoestrogenic conditions. 16α-OHE1 has been found to be elevated in strains of mice susceptible to breast cancer (11). In humans, estrogen metabolism has also been primarily studied in relation to female breast cancer risk (12-17, 33-36).

There are a number of potential explanations for our findings regarding prostate cancer risk, including potential effects of the neoplastic tissue on estrogen metabolism. Prostate cancer tissues and cells are equipped with key enzymes of estrogen metabolism, including hydroxylases, whose activity varies according to estrogen receptor status and responsiveness (37). The differences we observed may be related to the disease processes themselves rather than to etiological differences (37-40).

Another possible source of bias in our study may be related to selection of cases and controls. Because in the studied community prostate cancer is often diagnosed and treated by a large number of private physicians, we were not able to conduct a population-based study. A primary reason for this restriction was that the study entailed specimen collection prior any treatment. As a result, our cases were limited to those attending the largest hospitals in the area, the Department of Veterans Affairs Medical Center of Western New York Health Care System (VAMC) and from Kaleida Health System (Buffalo General Hospital). Our control subjects were selected among residents in Erie and Niagara Counties. Differences in the two populations could bias our results. In order to limit, at least in part, the potential effects of lifestyle bias, the control

subjects were matched on area of residence (neighborhood controls). In addition, because we still observed relevant differences in degree of education between prostate cancer cases and control subjects, we further adjusted for education in the analyses.

In the present study urinary estrogen metabolite levels were determined controlling for several sources of hormone variability both by inclusion criteria and highly standardized conditions at urine collection. Urine samples were collected from prostate cancer cases before cancer treatment was begun, and control subjects were evaluated for potential presence of latent prostate cancer by serum analysis for prostate-specific antigen. Prostate cancer cases and controls with conditions that would alter hormone metabolism were excluded. Care was taken to control for circadian variation in hormone levels. All hormone determinations were performed at the end of the study to reduce technical variability. The laboratory assaying estrogen metabolites was blinded to case-control status. Thus, we minimized potential biases in urine collection and hormone level determination.

In conclusion, this study supports the hypothesis that there is difference in the way estrogens are metabolized between patients affected with prostate cancer and control subjects. Further studies are needed to corroborate these findings that offer the possibility of a new research perspective on role of hormones in prostate cancer development.

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